

## BERESKIN &amp; PARR

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(REV. 11-2000)

U.S. DEPARTMENT OF COMMERCE, PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER

6580-273

TRANSMITTAL RETURN TO THE UNITED STATES  
DESIGNATED/ELECTED OFFICE (DO/EO/US)  
CONCERNING A FILING UNDER 35 U.S.C. 371

U.S. APPLICATION NO. (If known, see 37 CFR 1.5

09/980706

INTERNATIONAL APPLICATION NO.

PCT/CA00/00716

INTERNATIONAL FILING DATE

June 14, 2000

PRIORITY DATE CLAIMED

June 15, 1999

TITLE OF INVENTION

A Marker Specific For Escherichia Coli Serotypes O157:H7; O157:NM and O55:H7

APPLICANT(S) FOR DO/EO/US

Shu CHEN, Renlin XU, Jiping LI

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. ☐ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
  - a. ☐ is attached hereto (required only if not communicated by the International Bureau).
  - b. ☒ has been communicated by the International Bureau.
  - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
  - a. ☐ is attached hereto.
  - b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
  - a. ☐ are attached hereto (required only if not communicated by the International Bureau).
  - b. ☐ have been communicated by the International Bureau.
  - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
  - d. ☐ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 20 below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A **FIRST** preliminary amendment.
14. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
15. ☐ A substitute specification.
16. ☐ A change of power of attorney and/or address letter.
17. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
18. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. ☐ Other items or information:

U.S. APPLICATION NO. (if known, see 37 CFR 1.5)

INTERNATIONAL APPLICATION NO  
PCT/CA00/00716ATTORNEY'S DOCKET NUMBER  
6580-273

09/980706

21. ☒ The following fees are submitted:**BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):**Neither international preliminary examination fee (37 CFR 1.482)  
nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO  
and International Search Report not prepared by the EPO or JPO. .... \$1000.00International preliminary examination fee (37 CFR 1.482) not paid to  
USPTO but International Search Report prepared by the EPO or JPO ..... \$860.00International preliminary examination fee (37 CFR 1.482) not paid to USPTO  
but international search fee (37 CFR 1.445(a)(2)) paid to USPTO ..... \$710.00International preliminary examination fee (37 CFR 1.482) paid to USPTO  
but all claims did not satisfy provisions of PCT Article 33(1)-(4) ..... \$690.00International preliminary examination fee (37 CFR 1.482) paid to USPTO  
and all claims satisfied provisions of PCT Article 33(1)-(4) ..... \$100.00**ENTER APPROPRIATE BASIC FEE AMOUNT =**

## CALCULATIONS PTO USE ONLY

\$ 890.00

Surcharge of \$130.00 for furnishing the oath or declaration later than ☐ 20 ☐ 30  
months from the earliest claimed priority date (37 CFR 1.492(e)).

\$

CLAIMS NUMBER FILED NUMBER EXTRA RATE

Total claims - 20 = x \$18.00

Independent claims - 3 = x \$80.00

MULTIPLE DEPENDENT CLAIM(S) (if applicable) + \$270.00

**TOTAL OF ABOVE CALCULATIONS =**

\$ 890.00

☒ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above  
are reduced by 1/2. +

\$ 445.00

**SUBTOTAL =**

\$ 445.00

Processing fee of \$130.00 for furnishing the English translation later than ☐ 20 ☐ 30  
months from the earliest claimed priority date (37 CFR 1.492(f)).

\$

**TOTAL NATIONAL FEE =**

\$ 445.00

Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be  
accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property +

\$

**TOTAL FEES ENCLOSED =**

\$ 445.00

Amount to be  
refunded:

\$

charged:

\$

- a. ☒ A check in the amount of \$ 445.00 to cover the above fees is enclosed. # 4016
- b. ☐ Please charge my Deposit Account No. \_\_\_\_\_ in the amount of \$ \_\_\_\_\_ to cover the above fees.  
A duplicate copy of this sheet is enclosed.
- c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any  
overpayment to Deposit Account No. 022095 / A duplicate copy of this sheet is enclosed.
- d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. Credit card  
information should not be included on this form. Provide credit card information and authorization on PTO-2038.

**NOTE:** Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR  
1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Bereskin & Parr  
Box 401, 40 King Street West  
Toronto, Ontario Canada M5H 3Y2

SIGNATURE

MICHELINE GRAVELLE

NAME

40,261

REGISTRATION NUMBER

Barristers and Solicitors/Patent and Trade Mark Agents  
Practice Restricted to Intellectual Property Law

December 6, 2001

Micheline Gravelle B.Sc., M.Sc. (Immunol.)  
416 957 1682 mgravelle@bereskinparr.com

Your Reference: n/a  
Our Reference: 6580-273

Commissioner for Patents and Trademarks  
Washington, D.C. 20231  
U.S.A.

Dear Sirs:

**Re: PRELIMINARY AMENDMENT**  
**United States National Phase Entry of PCT/CA00/00716**  
**Entitled: A Marker Specific For Escherichia Coli Serotypes**  
**O157:H7; O157:NM and O55:H7**  
**Inventors: Shu Chen, Renlin Xu, Jiping Li**

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We are simultaneously entering national phase in the United States for PCT/CA00/00716. The present letter is to file a Preliminary Amendment to the application. Please amend the application as follows:

**In the Claims:**

Please amend claims 3, 5, 10, 11, 13, 14, 19 and 20 as follows:

3. (Amended) An isolated nucleic acid primer comprising (a) a portion of a sequence as claimed in claim 1 or (b) a sequence that is complimentary to a portion of a nucleic acid sequence claimed in claim 1.

5. (Amended) An isolated nucleotide probe comprising a portion of a sequence as claimed in claim 1.

10. (Amended) A method of detecting the presence of E. coli serotypes O157:H7; O157:NM or O55:H7 in a sample comprising (a) isolating nucleic acid from the sample and (b) determining if the isolated nucleic acid contains a nucleic acid

sequence according to claim 1, wherein the presence of a nucleic acid sequence according to claim 1 indicates the presence of E. coli serotypes O157:H7; O157:NM or O55:H7 in the sample.

11. (Amended) A method of detecting the presence of E. coli serotypes O157:H7; O157:NM or O55:H7 in a sample comprising (a) isolating nucleic acid from the sample; (b) amplifying the isolated nucleic acid with a primer having a sequence that is complimentary to a portion of a nucleic acid sequence according to claim 1 and (c) assaying for amplified sequences, wherein the presence of an amplified sequence indicates that the sample contains E. coli serotypes O157:H7; O157:NM or O55:H7.

13. (Amended) A method according to claim 11 wherein the nucleic acid is amplified in step (b) using a Polymerase Chain Reaction.

14. (Amended) A method of detecting the presence of a nucleic acid molecule associated with E. coli serotypes O157:H7; O157:NM and O55:H7 in a sample comprising (a) contacting the sample under hybridization conditions with one or more of nucleotide probes which hybridize to nucleic acid molecules according to claim 1 and (b) determining if there is hybridization between the nucleic acid molecules in the sample and the nucleotide probes, wherein the presence of hybridization indicates that the sample contains E. coli serotypes O157:H7; O157:NM or O55:H7.

19. (Amended) A microchip comprising a nucleic acid molecule according to claim 1 attached to a microchip.

20. (Amended) A microchip comprising a nucleotide probe according to claim 5 attached to a microchip.

**REMARKS**

By the present amendment, claims 3, 5, 10, 11, 13, 14, 19 and 20 have been amended in order to delete multiple dependencies. The Preliminary Amendment does not contain new matter.

Entry of the above preliminary amendment is respectfully requested. Please calculate the claim fee for the application once the amendment has been entered.

Attached hereto is a marked-up version of the changes made to the claims by the current amendment. The attached page is captioned "Version with markings to show changes made".

Respectfully submitted,

**Shu Chen, Renlin Xu, Jiping Li**



Micheline Gravelle  
Registration No. 40,261

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~~Toronto, Ontario, Canada~~  
~~M5H 3Y2~~

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**VERSION WITH MARKINGS TO SHOW CHANGES MADE**

**In the Claims:**

Claims 3, 5, 10, 11, 13, 14, 19 and 20 have been amended as follows:

3. (Amended) An isolated nucleic acid primer comprising (a) a portion of a sequence as claimed in claim 1 [or 2] or (b) a sequence that is complimentary to a portion of a nucleic acid sequence claimed in claim 1 [or 2].

5. (Amended) An isolated nucleotide probe comprising a portion of a sequence as claimed in claim 1 [or 2].

10. (Amended) A method of detecting the presence of E. coli serotypes O157:H7; O157:NM or O55:H7 in a sample comprising (a) isolating nucleic acid from the sample and (b) determining if the isolated nucleic acid contains a nucleic acid sequence according to claim 1 [or 2], wherein the presence of a nucleic acid sequence according to claim 1 [or 2] indicates the presence of E. coli serotypes O157:H7; O157:NM or O55:H7 in the sample.

11. (Amended) A method of detecting the presence of E. coli serotypes O157:H7; O157:NM or O55:H7 in a sample comprising (a) isolating nucleic acid from the sample; (b) amplifying the isolated nucleic acid with a primer having a sequence that is complimentary to a portion of a nucleic acid sequence according to claim 1 [or 2] and (c) assaying for amplified sequences, wherein the presence of an amplified sequence indicates that the sample contains E. coli serotypes O157:H7; O157:NM or O55:H7.

13. (Amended) A method according to claim 11 [or 12] wherein the nucleic acid is amplified in step (b) using a Polymerase Chain Reaction.

14. (Amended) A method of detecting the presence of a nucleic acid molecule associated with E. coli serotypes O157:H7; O157:NM and O55:H7 in a sample comprising (a) contacting the sample under hybridization conditions with one or more of nucleotide probes which hybridize to nucleic acid molecules according to claim 1 [or 2] and (b) determining if there is hybridization between the nucleic acid molecules in the sample and the nucleotide probes, wherein the presence of hybridization indicates that the sample contains E. coli serotypes O157:H7; O157:NM or O55:H7.

19. (Amended) A microchip comprising a nucleic acid molecule according to claim 1 [or 2] attached to a microchip.

20. (Amended) A microchip comprising a nucleotide probe according to [any one of] claim[s] 5 [to 9] attached to a microchip.

**Bereskin & Parr**  
**09/980706**

Barristers and Solicitors/Patent and Trade Mark Agents  
 Practice Restricted to Intellectual Property Law

Micheline Gravelle B.Sc., M.Sc. (Immunol.)  
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Your Reference: 09/980,706  
 Our Reference: 6580-273

March 21, 2002



Commissioner for Patents  
 United States Patent & Trademark Office  
 Washington, D.C. 20231  
 U.S.A.

**Attention: Box PCT**

Dear Sir:

**Re: RESPONSE TO NOTIFICATION OF MISSING REQUIREMENTS UNDER  
 35 U.S.C. 371 IN THE UNITED STATES DESIGNATED/ELECTED  
 OFFICE (DO/EO/US)  
 United States Patent Application No. 09/980,706  
 Entitled: A Marker Specific for Escherichia Coli Serotypes 0157:H7;  
 0157:NM and 055:H7  
 Inventors: Shu Chen, Renlin Xu and Jiping Li  
 Filing Date: June 14, 2000**

This is in response to the Notification of Missing Requirements mailed January 23, 2002, a copy of which we attach.

Please amend the application as follows:

**In the Specification**

Please replace Sequence Listing pages 1/2-2/2 currently of record with Sequence Listing pages 27-29 enclosed herewith.

**In the Claims**

Please renumber claim pages 27-30 currently of record as pages 30-33.

**In the Abstract**

Please renumber the abstract page as page 34.



## REMARKS

### Declaration

In accordance with the provisions of 37 C.F.R. §1.497(a) and (b), Applicants hereby submit an executed Declaration for Patent Application of inventors **Shu Chen, Renlin Xu and Jiping Li**. Applicants also enclose a Supplemental Application Data Sheet which includes markings to show the changes made from the Initial Application Data Sheet.

### Fees

The government fee of \$65.00 (small entity) is included in our cheque No. 4260, in respect of the surcharge for providing the declaration later than 30 months from the priority date.

If any additional fee is due, including a further fee for an extension of time, such an extension is hereby requested and the Commissioner is authorized to charge any such fee to Deposit Account No. 02-2095.

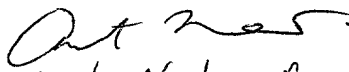
### Sequence Listing


In order to comply with the requirements of 37 C.F.R. 1.821-1.825, Applicants are submitting herewith (1) a Sequence Listing in paper form; (2) a Sequence Listing in computer readable form (a 3.5" floppy diskette) in the ASCII format and (3) a statement (set forth below) that the paper form and the computer readable form of the Sequence Listing are the same.

In accordance with the requirements of 37 C.F.R. 1.821-1.825 the undersigned verifies that the paper form of the Sequence Listing and the computer readable form of the Sequence Listing are the same. No new matter has been added.

Respectfully submitted,

**Shu Chen, Renlin Xu and Jiping Li**

  
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**VERSION WITH MARKINGS TO SHOW CHANGES MADE**

**In the Specification**

Sequence Listing pages 27-29 have replaced Sequence Listing pages 1/2-2/2.

**In the Claims**

Claim pages 27-30 have been renumbered as pages 30-33.

**In the Abstract**

Abstract page has been renumbered as page 34.

SEQUENCE LISTING

<110> Chen, Shu

Xu, Renlin

Li, Jiping

<120> A Marker Specific for Escherichia Coli Serotypes 0157:H7; 0157:NM and 055:H7

<130> 6580-273

<140> US 09/980,706

<141> 2000-06-14

<150> US 60/139,260

<151> 1999-06-15

<160> 8

<170> PatentIn version 3.1

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19

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60

acagctatcg agtaaaccac c

81

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<213> E. coli

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96

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<212> DNA

<213> E. coli

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17

<210> 8

<211> 17

<212> DNA

<213> E. coli

<400> 8

gatgagtcct gagtaag

17

09/980706

**Title:** A marker specific for *Escherichia coli* serotypes O157:H7; O157:NM and O55:H7

#### **FIELD OF THE INVENTION**

5                   This invention relates to a novel DNA marker specific for *E. coli* serotypes O157:H7, O157:NM and O55:H7 and the use of the marker in developing assays to detect these serotypes of *E. coli* in a sample.

#### **BACKGROUND OF THE INVENTION**

10                   *E. coli* O157:H7 is a food-borne human pathogen causing a spectrum of diseases including diarrhea, hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS). Development of nucleic-based assays for rapid detection of *E. coli* O157:H7 has been challenging due to a lack of specific DNA markers for this organism. The reported DNA markers for *E. coli* O157:H7 have one or more of the following limitations or  
15 drawbacks: being non-specific, so that multiplex PCR assays need to be used; and containing only one base pair mutation, limiting their use in assay development. Consequently, there is a need in the art to provide novel markers for *E. coli* O157:H7 and related serotypes.

#### **SUMMARY OF THE INVENTION**

20                   The present inventors have prepared a novel marker specific for *E. coli* serotypes O157:H7, O157:NM and O55:H7. The DNA sequence of the marker has a total of 1583 nucleotides and has no significant homology to any known DNA sequences. Accordingly, in one aspect, the present invention provides an isolated nucleic acid sequence comprising the  
25 sequence shown in Figure 1 (SEQ.ID.NO.:1) or a diagnostic fragment thereof.

                  The marker of the invention can be used to develop probes or primers that can be used to detect *E. coli* serotypes O157:H7; O157:NM and O55:H7 in a sample. In particular, the marker has been demonstrated to be  
30 useful in designing primers for PCR assays for specific detection of the *E. coli* serotypes. As the specific DNA sequences of this invention are not homologous to previously known sequences, various specific PCR assays

- 2 -

can be developed with only one primer pair. In addition, other nucleic acid-based assays such as DNA chip or biosensor assays can also be developed without the restriction in using a very limited region of a marker. Therefore, the invention can be used to develop nucleic acid based assays to detect the *E. coli* serotypes O157:H7, O157:NM and O55:H7.

Other features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

#### **BRIEF DESCRIPTION OF THE DRAWINGS**

The invention will now be described in relation to the drawings in which:

Figure 1 and SEQ.ID.NO.:1 shows the nucleotide sequence of the 1583 bp marker of the invention.

Figure 2 and SEQ.ID.NO.:2 shows the nucleotide sequence of a 360 bp fragment of the marker.

Figure 3 is a gel electrophoresis of PCR products amplified using the PCR assay from DNA preparations of representative *E. coli* serotypes: lanes 1, 2, O157:H7; lanes 3, 4, O157:NM; lanes 5 - 9, O145:NM; lanes 10, 11, O55:H7; lane 12, O26:H11; lane M, 100 bp DNA ladder.

#### **DETAILED DESCRIPTION OF THE INVENTION**

##### **I. MARKER**

As hereinbefore mentioned, the present inventors have prepared a novel DNA marker specific for *E. coli* serotypes O157:H7, O157:NM and O55:H7.

The marker was identified and isolated using a relatively new marker technology, fluorescent amplified fragment length polymorphism (FAFLP) and DNA sequencing. The DNA sequence of the marker has a

- 3 -

total of 1583 nucleotides and has no significant homology to any known DNA sequences.

Accordingly, in one aspect, the present invention provides an isolated nucleic acid molecule comprising the sequence shown in Figure 1 (SEQ.ID.NO.:1), wherein T can also be U, or a diagnostic fragment thereof.

The term "isolated" refers to a nucleic acid molecule substantially free of cellular material or culture medium when produced by recombinant DNA techniques or chemical precursors when chemically synthesized. The term "nucleic acid" includes deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) and can either be double stranded or single stranded.

The term "diagnostic fragment" means any fragment of the marker shown in Figure 1 (SEQ.ID.NO.:1) that is useful in a diagnostic assay to detect E. coli serotypes O157:H7; O157:NM and O55:H7. The diagnostic fragment includes fragments that can be used as primers in PCR assays and fragments that can be used as probes in detection assays. The diagnostic fragments of the invention will not cross react with bacteria other than E. coli serotypes O157:H7; O157:NM and O55:H7.

The invention also includes (1) a nucleic sequence that is complementary to the sequence shown in Figure 1 (SEQ.ID.NO.:1) or a diagnostic fragment thereof; (2) a nucleic sequence that can hybridize to the sequence shown in Figure 1 (SEQ.ID.NO.:1) or a diagnostic fragment thereof; (3) a nucleic acid sequence which has substantial sequence homology to (1) or (2); and (4) a nucleic acid sequence which is an analog of the nucleic acid sequences of (1) to (3). Such sequences are also useful in developing diagnostic assays to detect E. coli serotypes O157:H7; O157:NM and O55:H7.

The term "a nucleic acid sequence which has substantial sequence homology" means a nucleic acid sequence which has slight or inconsequential sequence variations from the sequence shown in Figure 1 (SEQ.ID.NO.:1). For example, one skilled in the art can appreciate that variations can be made to the sequence of Figure 1 (SEQ.ID.NO.:1) and still



- 4 -

permit the use of the sequence in detecting the E. coli serotypes of the invention. In addition, natural variations may exist in the sequence of certain isolates which may be attributable to local mutations or structural modifications. Nucleic acid sequences having substantial homology  
5 include nucleic acid sequences having at least 85%, preferably 90-95% identity with the nucleic acid sequence as shown in Figure 1 (SEQ.ID.NO.:1).

The term "a nucleic acid sequence that can hybridize" means a nucleic acid sequence that can hybridize to a nucleic acid sequence shown  
10 in Figure 1 (SEQ.ID.NO.:1) or a diagnostic fragments thereof under hybridization conditions, preferably stringent hybridization conditions. Appropriate stringency conditions which promote DNA hybridization are known to those skilled in the art, or may be found in Current Protocols in Molecular Biology, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. For  
15 example, the following may be employed: 6.0 x sodium chloride/sodium citrate (SSC) at about 45°C, followed by a wash of 2.0 x SSC at 50°C. The stringency may be selected based on the conditions used in the wash step. For example, the salt concentration in the wash step can be selected from a high stringency of about 0.2 x SSC at 50°C. In addition, the temperature in  
20 the wash step can be at high stringency conditions, at about 65°C.

The term "a nucleic acid sequence which is an analog" means a nucleic acid sequence which has been modified as compared to the sequence shown in Figure 1 (SEQ.ID.NO.:1) or a diagnostic fragment thereof wherein the modification does not alter the utility of the sequence  
25 (i.e. to detect E. Coli serotypes O157:H7; O157:NM and O55:H7 or to develop probes, primers or microchips to detect the serotypes) as described herein. The modified sequence or analog may have improved properties over the sequence shown in Figure 1 (SEQ.ID.NO.:1) or a diagnostic fragment thereof. One example of a modification to prepare an analog is to replace  
30 one of the naturally occurring basis (i.e. adenine, guanine, cytosine or thymidine) of the sequence shown in Figure 1 (SEQ.ID.NO.:1) with a modified base such as such as xanthine, hypoxanthine, 2-aminoadenine, 6-

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methyl, 2-propyl and other alkyl adenines, 5-halo uracil, 5-halo cytosine, 6-aza uracil, 6-aza cytosine and 6-aza thymine, pseudo uracil, 4-thiouracil, 8-halo adenine, 8-aminoadenine, 8-thiol adenine, 8-thiolalkyl adenines, 8-hydroxyl adenine and other 8-substituted adenines, 8-halo guanines, 8-amino guanine, 8-thiol guanine, 8-thiolalkyl guanines, 8-hydroxyl guanine and other 8-substituted guanines, other aza and deaza uracils, thymidines, cytosines, adenines, or guanines, 5-trifluoromethyl uracil and 5-trifluoro cytosine.

Another example of a modification is to include modified phosphorous or oxygen heteroatoms in the phosphate backbone, short chain alkyl or cycloalkyl intersugar linkages or short chain heteroatomic or heterocyclic intersugar linkages in the nucleic acid molecule shown in Figure 1 (SEQ.ID.NO.:1). For example, the nucleic acid sequences may contain phosphorothioates, phosphotriesters, methyl phosphonates, and phosphorodithioates.

A further example of an analog of a nucleic acid molecule of the invention is a peptide nucleic acid (PNA) wherein the deoxyribose (or ribose) phosphate backbone in the DNA (or RNA), is replaced with a polyamide backbone which is similar to that found in peptides (P.E. Nielsen, et al Science 1991, 254, 1497). PNA analogs have been shown to be resistant to degradation by enzymes and to have extended lives *in vivo* and *in vitro*. PNAs also bind stronger to a complimentary DNA sequence due to the lack of charge repulsion between the PNA strand and the DNA strand. Other nucleic acid analogs may contain nucleotides containing polymer backbones, cyclic backbones, or acyclic backbones. For example, the nucleotides may have morpholino backbone structures (U.S. Pat. No. 5,034,506). The analogs may also contain groups such as reporter groups, a group for improving the pharmacokinetic or pharmacodynamic properties of nucleic acid sequence.

Other analogs include isolated nucleic acid sequences having sequences which differ from the nucleic acid sequence shown in Figure 1 (SEQ.ID.NO.:1) due to degeneracy in the genetic code are also within the

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scope of the invention. Such nucleic acids encode functionally equivalent proteins but differ in sequence from the above mentioned sequences due to degeneracy in the genetic code.

The present invention further includes the preparation or  
5 isolation of other nucleic acid sequences which are the same, analogous, homologous or can hybridize to the nucleic acid sequences of the invention. For example, an isolated nucleic acid molecule of the invention which comprises DNA can be isolated by preparing a labelled nucleic acid probe based on all or part of the nucleic acid sequences as  
10 shown in Figure 1 (SEQ.ID.NO.:1), and using this labelled nucleic acid probe to screen an appropriate DNA library (e.g. a cDNA or genomic DNA library). For example, a genomic library can be used to isolate a DNA by screening the library with the labelled probe using standard techniques. Nucleic acids isolated by screening of a cDNA or genomic DNA library can  
15 be sequenced by standard techniques.

An isolated nucleic acid molecule of the invention which is DNA can also be isolated by selectively amplifying a nucleic acid using the polymerase chain reaction (PCR) methods and cDNA or genomic DNA. It is possible to design synthetic oligonucleotide primers from the nucleic  
20 acid sequence as shown in Figure 1 (SEQ.ID.NO.:1), for use in PCR. A nucleic acid can be amplified from cDNA or genomic DNA using these oligonucleotide primers and standard PCR amplification techniques. The nucleic acid so amplified can be cloned into an appropriate vector and characterized by DNA sequence analysis. It will be appreciated that cDNA  
25 may be prepared from mRNA, by isolating total cellular mRNA by a variety of techniques, for example, by using the guanidinium-thiocyanate extraction procedure of Chirgwin et al., Biochemistry, 18, 5294-5299 (1979). cDNA is then synthesized from the mRNA using reverse transcriptase (for example, Moloney MLV reverse transcriptase available from Gibco/BRL, Bethesda, MD, or AMV reverse transcriptase available from Seikagaku  
30 America, Inc., St. Petersburg, FL).

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An isolated nucleic acid molecule of the invention which is RNA can be isolated by cloning a cDNA of the invention into an appropriate vector which allows for transcription of the cDNA to produce an RNA molecule. For example, a cDNA can be cloned downstream of a bacteriophage promoter, (e.g., a T7 promoter) in a vector, cDNA can be transcribed *in vitro* with T7 polymerase, and the resultant RNA can be isolated by standard techniques.

A nucleic acid molecule of the invention may also be chemically synthesized using standard techniques. Various methods of chemically synthesizing polydeoxynucleotides are known, including solid-phase synthesis which, like peptide synthesis, has been fully automated in commercially available DNA synthesizers (See e.g., Itakura et al. U.S. Patent No. 4,598,049; Caruthers et al. U.S. Patent No. 4,458,066; and Itakura U.S. Patent Nos. 4,401,796 and 4,373,071).

The sequence of a nucleic acid molecule of the invention may be inverted relative to its normal presentation for transcription to produce an antisense nucleic acid molecule. Preferably, an antisense sequence is constructed by inverting a region preceding the initiation codon or an unconserved region. In particular, the nucleic acid sequences contained in the nucleic acid molecules of the invention or a fragment thereof, preferably a nucleic acid sequence shown in Figure 1 (SEQ.ID.NO.:1) may be inverted relative to its normal presentation for transcription to produce antisense nucleic acid molecules.

The antisense nucleic acid molecules of the invention or a fragment thereof, may be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed with mRNA or the native gene e.g. phosphorothioate derivatives and acridine substituted nucleotides. The antisense sequences may be produced biologically using an expression vector introduced into cells in the form of a recombinant plasmid, phagemid or attenuated virus in which antisense sequences are produced under the control of a high

efficiency regulatory region, the activity of which may be determined by the cell type into which the vector is introduced.

## II. USES OF THE MARKER

As previously mentioned, the isolation of the novel marker  
5 for *E. coli* serotypes O157:H7; O157:NM and O55:H7 allows the development of diagnostic assays that can be used to detect the serotypes in a sample. The sample can be any sample, including but not limited to, clinical, food, water and environmental samples. Clinical samples include bodily materials such as blood, urine, serum, tears, saliva, feces, tissues and  
10 the like.

Accordingly, the present invention provides a method of detecting the presence of *E. coli* serotypes O157:H7; O157:NM or O55:H7 in a sample comprising (a) isolating nucleic acid from the sample and (b) determining if the isolated nucleic acid contains (1) a nucleic sequence  
15 shown in Figure 1 (SEQ.ID.NO.:1) wherein T can also be U or a diagnostic fragment thereof; (2) a nucleic sequence that is complimentary to the sequence shown in Figure 1 (SEQ.ID.NO.:1) or a diagnostic fragment thereof; (3) a nucleic sequence that can hybridize to the sequence shown in Figure 1 (SEQ.ID.NO.:1) or a diagnostic fragment thereof; (4) a nucleic acid  
20 sequence which has substantial sequence homology to (1), (2) or (3); or (5) a nucleic acid sequence which is an analog of any of the nucleic acid sequences of (1) to (4), wherein the presence of a nucleic acid sequence defined in (1) to (5) indicates the presence of *E. coli* serotypes O157:H7; O157:NM or O55:H7 in the sample.

### 25 (a) Primers

The present invention includes the preparation of nucleic acid primers based on the sequence of the marker shown in Figure 1 (SEQ.ID.NO.:1). Accordingly, the present invention provides an isolated nucleic acid primer comprising a portion of a (1) a nucleic sequence shown  
30 in Figure 1 (SEQ.ID.NO.:1) wherein T can also be U or a diagnostic fragment thereof; (2) a nucleic sequence that is complimentary to the sequence shown in Figure 1 (SEQ.ID.NO.:1) or a diagnostic fragment

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thereof; (3) a nucleic sequence that can hybridize to the sequence shown in Figure 1 (SEQ.ID.NO.:1) or a diagnostic fragment thereof; (4) a nucleic acid sequence which has substantial sequence homology to (1), (2) or (3); or (5) a nucleic acid sequence which is an analog of any of the nucleic acid sequences of (1) to (4). Preferably, the primer contains from about 5 to about 50 nucleotides, more preferably from about 15 to 30 nucleotides.

The length and bases of primers are selected so that they will hybridize to different strands of the sequence as shown in Figure 1 (SEQ.ID.NO.:1) and at relative positions along the sequence such that an extension product synthesized from one primer when it is separated from its template can serve as a template for extension of the other primer into a nucleic acid of defined length. Primers which may be used in the invention are oligonucleotides, i.e., molecules containing two or more deoxyribonucleotides of the nucleic acid molecule of the invention which occur naturally as in a purified restriction endonuclease digest or are produced synthetically using techniques known in the art such as for example phosphotriester and phosphodiester methods (See Good *et al.* Nucl. Acid Res 4:2157, 1977) or automated techniques (See for example, Conolly, B.A. Nucleic Acids Res. 15:15(7): 3131, 1987). The primers are capable of acting as a point of initiation of synthesis when placed under conditions which permit the synthesis of a primer extension product which is complementary to a DNA sequence of the invention, i.e., in the presence of nucleotide substrates, an agent for polymerization such as DNA polymerase and at suitable temperature and pH. Preferably, the primers are sequences that do not form secondary structures by base pairing with other copies of the primer or sequences that form a hair pin configuration.

It will be appreciated that the primers may contain non-complementary sequences provided that a sufficient amount of the primer contains a sequence which is complementary to a nucleic acid molecule of the invention or oligonucleotide fragment thereof, which is to be amplified. Restriction site linkers may also be incorporated into the

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primers allowing for digestion of the amplified products with the appropriate restriction enzymes facilitating cloning and sequencing of the amplified product.

In one embodiment, the primer comprises nucleotides 597-  
5 618 of the sequence shown in Figure 1 (SEQ.ID.NO.:1). In another  
embodiment, the primer comprises the reverse complement of  
nucleotides 1136-1155 of the sequence shown in Figure 1 (SEQ.ID.NO.:1).  
In a specific embodiment, the primer is O157-F and has the sequence 5'-  
CGGTTTAATGGCTTGTGCT-3' (SEQ.ID.NO.:3). This primer  
10 corresponds to the sequence at nucleotides 597-618 of SEQ.ID.NO.:1 except  
that the G residue at position 617 has been replaced with C in the primer.  
In another specific embodiment, the primer is O157-R and has the  
sequence 5'-ATGCCATTAAACCGGTGGC-3' (SEQ.ID.NO.:4). This primer  
corresponds to the reverse complement of the nucleotides found at  
15 positions 1136-1155 of SEQ.ID.NO.:1. These are shown in Table 3.

Using the primers illustrated in Table 3 in a PCR assay, the  
inventors have demonstrated that these primers are specific for the E. coli  
serotypes O157:H7; O157:NM and O55:H7 but are not specific for 119 other  
E. coli strains belonging to 60 serotypes and 59 isolates belonging to 44 non-  
20 E. coli species (see Example 1).

Accordingly, the present invention provides a method of  
detecting the presence of E. coli serotypes O157:H7; O157:NM and O55:H7  
in a sample comprising (a) isolating nucleic acid from the sample;  
(b) amplifying the isolated nucleic acid with a primer comprising a  
25 sequence that is complimentary to a portion of (1) a nucleic sequence  
shown in Figure 1 (SEQ.ID.NO.:1) wherein T can also be U or a diagnostic  
fragment thereof; (2) a nucleic sequence that is complimentary to the  
sequence shown in Figure 1 (SEQ.ID.NO.:1) or a diagnostic fragment  
thereof; (3) a nucleic sequence that can hybridize to the sequence shown in  
30 Figure 1 (SEQ.ID.NO.:1) or a diagnostic fragment thereof; (4) a nucleic acid  
sequence which has substantial sequence homology to (1), (2) or (3); or (5) a  
nucleic acid sequence which is an analog of any of the nucleic acid

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sequences of (1) to (4); and (c) assaying for amplified sequences, wherein the presence of an amplified sequence indicates that the sample contains one or more of E. coli serotypes O157:H7; O157:NM and O55:H7. Preferably, a primer pair is used, one with a sequence shown in SEQ.ID.NO.:3 and the  
5 other with a sequence shown in SEQ.ID.NO.:4.

The nucleic acid sequences may be amplified in step (b) using any method that results in the amplification of nucleic acid molecules such as the Polymerase Chain Reaction, Ligase Chain Reaction or NASBA may be used to amplify a nucleic acid molecule of the invention (Barney  
10 in "PCR Methods and Applications", August 1991, Vol.1(1), page 5, and European Published Application No. 0320308, published June 14, 1989, and U.S. Serial No. 5,130,238 to Malek).

Preferably, the nucleic acid sequences are amplified in step (b) using a Polymerase Chain Reaction (PCR). The conditions which may be  
15 employed in the methods of the invention using PCR are those which permit hybridization and amplification reactions to proceed in the presence of DNA in a sample and appropriate complementary hybridization primers. Conditions suitable for the polymerase chain reaction are generally known in the art. For example, see M.A. Innis and  
20 D.H. Gelfand, PCR Protocols, A guide to Methods and Applications M.A. Innis, D.H. Gelfand, J.J. Sninsky and T.J. White eds, pp3-12, Academic Press 1989, which is incorporated herein by reference. Preferably, the PCR utilizes polymerase obtained from the thermophilic bacterium *Thermus aquaticus* (Taq polymerase, GeneAmp Kit, Perkin Elmer Cetus) or other  
25 thermostable polymerase may be used to amplify DNA template strands.

The amplified products can be isolated and distinguished based on their respective sizes using techniques known in the art. For example, after amplification, a DNA sample can be separated on an agarose gel and visualized, after staining with ethidium bromide, under ultra  
30 violet (uv) light. DNA may be amplified to a desired level and a further extension reaction may be performed to incorporate nucleotide derivatives having detectable markers such as radioactive labelled or biotin labelled



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nucleoside triphosphates. The primers may also be labelled with detectable markers as discussed below. The detectable markers may be analyzed by restriction and electrophoretic separation or other techniques known in the art.

5           The primers may be labelled with detectable markers which allow for detection of the amplified products. Suitable detectable markers are radioactive markers such as P-32, S-35, I-125, and H-3; luminescent markers such as chemiluminescent markers, preferably luminol; and  
10       and 4-fluor-7-nitrobenz-2-axa-1,3 diazole, Cy3, Cy5, Texas Red, NED; enzyme markers such as horseradish peroxidase, alkaline phosphatase,  $\beta$ -galactosidase, acetylcholinesterase, or biotin.

**(b) Probes**

          The present invention also includes the preparation of  
15       nucleic acid probes based on the sequence of the marker shown in Figure 1 (SEQ.ID.NO.:1). Accordingly, the present invention provides an isolated nucleotide probe comprising a portion of (1) a nucleic sequence shown in Figure 1 (SEQ.ID.NO.:1) wherein T can also be U or a diagnostic fragment thereof; (2) a nucleic sequence that is complimentary to the sequence  
20       shown in Figure 1 (SEQ.ID.NO.:1) or a diagnostic fragment thereof; (3) a nucleic sequence that can hybridize to the sequence shown in Figure 1 (SEQ.ID.NO.:1) or a diagnostic fragment thereof; (4) a nucleic acid sequence which has substantial sequence homology to (1), (2) or (3); or (5) a nucleic acid sequence which is an analog of any of the nucleic acid sequences of (1)  
25       to (4).

          Preferably, the probe comprises a portion of the sequence shown in Figure 1 (SEQ.ID.NO.:1). More preferably, the probe comprises from about 20 to about 500 nucleotides, even more preferably 50 to 200 nucleotides from the sequence shown in Figure 1 (SEQ.ID.NO.:1).

30           In one embodiment, the probe comprises nucleotides 597-677 of the sequence shown in Figure 1 (SEQ.ID.NO.:1). Preferably, the probe is O157-A and has the sequence CGGTTTAATGGCTTGTTGTGGTAA

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CACCGAAGCCAGCTCAATAAATTGCTGCGATGAGTTAC  
AGCTATCGAGTAAACCACC (SEQ.ID.NO.:5).

In another embodiment, the probe comprises nucleotides  
1059-1155 of Figure 1 (SEQ.ID.NO.:1). Preferably, the probe is O157-B and  
5 has the sequence TATCCCGAATCGCCTGGCGTTTTTGCACATC  
CTCTGCGACGCTATTTTTGTGGAACGCAAAGCCATCAAGGAAAATA  
GCCA CCGTTTAAT GGCAT (SEQ.ID.NO.:6).

The probes can be used to detect the presence or absence of E.  
coli serotypes O157:H7; O157:NM and O55:H7 in a sample.

10 Accordingly, the present invention also relates to a method of  
detecting the presence of a nucleic acid molecule associated with E. coli  
serotypes O157:H7; O157:NM and O55:H7 in a sample comprising  
(a) contacting the sample under hybridization conditions with one or  
more nucleotide probes which hybridize to the nucleic acid molecules and  
15 (b) determining if there is hybridization between the nucleic acid  
molecules in the sample and the nucleotide probes wherein the presence  
of hybridization indicates that the sample contains one of E. coli serotypes  
O157:H7; O157:NM and O55:H7.

Hybridization conditions which may be used in the methods  
20 of the invention are known in the art and are described for example above  
in the definition of "nucleic acid sequences that hybridize". The  
hybridization product may be assayed using techniques known in the art.  
The nucleotide probe may be labelled with a detectable marker and the  
hybridization product may be assayed by detecting the detectable marker or  
25 a detectable change produced by the detectable marker.

The detectable marker used to label the probe can be any  
marker such as a radioactive label which provides for an adequate signal  
and has sufficient half life such as <sup>32</sup>P, <sup>3</sup>H, <sup>14</sup>C or the like. Other detectable  
markers which may be used include antigens that are recognized by a  
30 specific labelled antibody, fluorescent compounds, enzymes, antibodies  
specific for a labelled antigen, and chemiluminescent compounds. An  
appropriate label may be selected having regard to the rate of hybridization

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and binding of the probe to the nucleotide to be detected and the amount of nucleotide available for hybridization.

**(c) Microchips**

The present invention also includes microchips comprising

5 (a) an isolated nucleic acid molecule comprising (1) a nucleic sequence shown in Figure 1 (SEQ.ID.NO.:1) wherein T can also be U or a diagnostic fragment thereof; (2) a nucleic sequence that is complimentary to the sequence shown in Figure 1 (SEQ.ID.NO.:1) or a diagnostic fragment thereof; (3) a nucleic sequence that can hybridize to the sequence shown in

10 Figure 1 (SEQ.ID.NO.:1) or a diagnostic fragment thereof; (4) a nucleic acid sequence which has substantial sequence homology to (1), (2) or (3); or (5) a nucleic acid sequence which is an analog of any of the nucleic acid sequences of (1) to (4) attached to (b) a microchip.

In one embodiment, the microchip comprises a probe having

15 the sequence CGGTTTAATGGCTTGTTGTGGTAACACCGAAGCCAGCTCAATAAATTGCTGCGATGAGTTACAGCTATCGAGTAAACCACC (SEQ.ID.NO.:5) attached to a microchip.

In another embodiment, the microchip comprises a probe having the sequence TATCCCGAATCGCCTGGCGTTTTTGCACATC

20 CTCTGCGACGCTATTTTTGTGGAACGCAAAGCCATCAAGGAAAATAGCCA CCGGTTTAATGGCAT (SEQ.ID.NO.:6) attached to a microchip.

As described in Example 2, microchips containing the probes shown in SEQ.ID.NO.:5 and SEQ.ID.NO.:6 were useful in detecting E. Coli serotype O157:H7.

25 **(d) Kits**

Reagents suitable for conducting the above described diagnostic methods of the invention may be packaged into convenient kits providing the necessary materials, packaged into suitable containers. Such kits may include all the reagents required to detect an E. coli serotype in a

30 sample by means of the methods described herein, such as appropriate probes and/or primers for performing PCR.

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In one embodiment of the invention the kit contains a nucleotide probe which hybridizes with a nucleic acid molecule of the invention, reagents required for hybridization of the nucleotide probe with the nucleic acid molecule, and directions for its use. In another embodiment of the invention, the kit includes primers which are capable of amplifying a nucleic acid molecule of the invention or a predetermined oligonucleotide fragment thereof, all the reagents required to produce the amplified nucleic acid molecule or predetermined fragment thereof in the polymerase chain reaction, and means for assaying the amplified sequences.

As mentioned above, the methods and kits of the present invention have many practical applications. For example, the methods and kits of the present invention may be used to detect E. Coli serotypes 0157:H7, 0157:NM or 055:H7 in any sample suspected of containing E. coli. Samples which may be tested include bodily materials such as blood, urine, serum, tears, saliva, feces, tissues and the like. Further, water and food samples and other environmental samples and industrial wastes may be tested.

Before testing a sample in accordance with the methods described herein, the sample may be concentrated using techniques known in the art, such as centrifugation and filtration. For hybridization and/or PCR-based methods described herein, nucleic acids may be extracted from cell extracts of the test sample using techniques known in the art such as precipitation, solvent extraction and column purification.

The following non-limiting examples are illustrative of the present invention:

### EXAMPLES

#### Example 1

##### *AFLP marker identification*

The marker was initially identified as a 360 bp fluorescent fragment (Figure 2 or SEQ.ID.NO.:2) using the FAFLP method (Vos et al., 1995. Nucleic Acids Research, Vol. 23, pp. 4407-4414). The FAFLP analysis

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was performed by the selective amplification of restriction fragments from a digest of total genomic DNA with restriction enzymes *EcoR* I and *Mse* I. The DNA sequences of the selective primer pair that produced the polymorphic marker are as shown in Table 1 and in SEQ.ID.NOS:7 and 8.

- 5 Among 163 *E. coli* strains tested that belonged to 40 serotypes (Table 2), only serotypes O157:H7, O157:NM, O145:NM and O55:H7 contained the 360 bp fragment.

#### *DNA sequencing*

- The target fragment was directly isolated from the AFLP gel using ABI 377 automated DNA Sequencer and re-amplified by polymerase chain reaction (PCR), and then sequenced. The DNA sequence of the 360 bp fragment is shown in Figure 2 (SEQ.ID.NO.:2). A 1223 bp downstream region continued from the AFLP fragment was further sequenced using a single primer walking method with strains of *E. coli* serotypes O157:H7, O157:NM, O55:H7 and O145:NM. Four mutations (C-T, A-G, T-A and G-A) in the region were identified in the strain of serotype O145:NM. The complete sequence of the 1583 bp fragment is shown in Figure 1 (SEQ.ID.NO.:1). The result of BLAST search showed that the DNA sequences of the 1583 bp fragment had no significant homology to 400 sequences of the *E. coli* genome and 400,635 known sequences in the GenBank, EMBL, DDBJ, PDB DNA databases. The DNA sequences of the 1583 bp fragment were also analyzed by a software program, GeneWorks 2.5; a potential open reading frame (ORF) was identified within the sequences of 1043 - 90 nt.

#### 25 *Marker application*

- The DNA fragment has been useful as a marker in developing a PCR assay for specific detection of *E. coli* serotypes O157:H7, O157:NM and O55:H7. The DNA sequences of the primers used in the PCR assay are provided in Table 3. Primer O157-F contains one mismatch to the sequences of O157:H7/O157:NM for eliminating possible cross reaction from the sequences of O145:NM. The primer pair results in a 560 bp PCR product under the following conditions. An amplification

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reaction mixture (20  $\mu$ L) contained 10mM Tris-HCl and 50 mM KCl (pH 8.3), 1.2 mM  $MgCl_2$ , 200  $\mu$ M each of dATP, dCTP, dGTP and dTTP, 0.4 pmol/ $\mu$ L each of the primers, 0.5 unit of *Taq* DNA polymerase (Perkin-Elmer) and 5  $\mu$ L of DNA template. The thermal cycling conditions (GeneAmp 9600 PCR System, Perkin-Elmer) were as follows: initial denaturation at 94°C for 3 min; 35 cycles consisting of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 45 sec; incubation at 72°C for 5 min. PCR products were visualized on a 1.6% agarose gel after staining with ethidium bromide.

The specificity of the primers and the PCR assay has been evaluated with a total of 119 *E. coli* strains belonging to 60 serotypes (Table 4) and 59 isolates belonging to 44 non-*E. coli* species (Table 5). The 560 bp specific PCR product appeared only with strains of serotypes O157:H7, O157:NM and O55:H7 (Figure 3) but not the strains of the other serotypes of *E. coli* or other bacterial strains tested. The PCR assay can be used for the detection of *E. coli* O157:H7 in food, clinical, water and environmental samples.

### Example 2

#### Preparation of a Microchip

## 20 MATERIALS AND METHODS

### *DNA microchip system*

The arrayer (with operation software) was a Brown Lab/Stanford System that was assembled at Virtek Vision (Waterloo, Ontario) and can produce 1000 to 2500 spots/cm<sup>2</sup>. The DNA scanner, ChipReader™ (with data collection software) was from Virtek Vision. The software ImaGene was the product of BioDiscovery and contributed by Virtek.

### *DNA probe preparation*

In this Example, "probe" refers to the DNA covalently attached to the chip surface and "target" in the fluorescent molecules that hybridize to this probe.

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The probes for *E. coli* serotype O157:H7 were designed according to the sequences of the O157:H7 marker and are illustrated in Table 6. Short (about 20 bp) probes were prepared by direct synthesis and 5'-end labeling with amine. Other probes were prepared by PCR using the

5 DNA samples from the control *E. coli* strains and specific primers with one of them labeled with 5'-amine.

PCR reaction mixtures (50 $\mu$ L) contain 1x amplification Buffer II (Perkin-Elmer), 200  $\mu$ M dNTPs, 2 mM  $MgCl_2$ , 0.5  $\mu$ M forward and reverse primers, 1 unit of AmpliTag DNA polymerase (Perkin-Elmer) and

10 10 $\mu$ L of template DNA. Amplification for probe preparation consisted of incubation at 95°C for 1 min. 32 cycles of 30s at 94°C, 30s at 52°C and 1 min at 72°C, and extension at 72°C for 7 min. PCR products were visualized using ethidium bromide-stained agarose gels, purified using QIAquick PCR purification Kit (Qiagen) and dissolved in 3x SSC buffer for spotting.

15 *Probe spotting and immobilization on the chips*

The purified DNA probes with amine-modified tags were spotted on a glass microscope slide that is coated with aldehyde groups (Telechem International, Inc.). The DNA probes were immobilized onto the glass surface by covalent binding via the Schiff's base attachment.

20 Spotting was carried out with the Stanford arrayer system. This robot system is designed to automatically collect samples (DNA probes) from either 96- or 384-well microtitre plates, and to spot the samples onto up to 16 slides simultaneously. The probes were spotted with a single pin at a density of 2500 spots/cm<sup>2</sup> (200  $\mu$ m spacing). After spotting, the slides were

25 rehydrated in a humidity chamber for 2-4 h, and rinsed once with 0.2% SDS and twice with water. The slides were then incubated for 5 min in a sodium borohydride solution (1.0%). The probes (if they were PCR products) were denatured by heating the slides at 95°C for 2 min, rinsing once with 0.2% SDS and twice with water. The slides were dried and

30 stored at room temperature until use.

### *Fluorescent target DNA preparation*

Fluorescent target DNAs were prepared by PCR from the control strains of bacteria with specific primers. The PCR reaction conditions were same as those used in the probe preparation except the following modifications: the primers were not labeled; 50% of the normal dCTP was substituted with 500  $\mu$ M of fluorescent Cy3-dCTP (Amersham Pharmacia Biotech) in the PCR reaction mixtures. The labeled target DNAs were purified with MicroSpin columns (Amersham Pharmacia Biotech) and stored at -20°C until use.

### 10 *DNA hybridization*

The immobilized probes were pre-hybridized with 1  $\mu$ g of salmon sperm DNA in 20  $\mu$ L of the DIG Easy Hyb solution (Boehringer Mannheim) at 65°C (37°C for small fragments) for 1 h. The target DNAs (10  $\mu$ L) were denatured at 95°C for 2 min, and mixed with 10  $\mu$ L of the DIG Easy Hyb solution and applied onto the processed slide with a coverslip. The slides were placed in a humid chamber and incubated overnight at 37°C or 62°C. Following hybridization, the slides were washed with the washing buffers with different stringency for four times and dried at room temperature.

### 20 *Data collection and analysis*

Hybridization patterns were scanned using the ChipReader™ (Virtek). Data were collected using the ChipReader™ collection software (Virtek) at 100% laser intensity, 700-900 detector sensitivity, 2 detector gain on the Cy-3 channel. Fluorescent images were analyzed using the ImaGene™ software (BioDiscovery).

### Results

The marker of the invention has been demonstrated to be useful in developing a DNA microarray/chip-based assay for specific detection of the *E. coli* serotypes O157:H7, O157:NM and O55:H7 in a sample. Table 6 shows the sequences of the two DNA probes that were designed based on the marker. The DNA probes suitable for spotting were



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generated using PCR and tagged with amine at their 5' end. The probes were used together with other 22 probes and immobilized onto the microscopic slide surface by covalent binding via Schiff's base attachment. The microarray was used in hybridization with Cy3 labeled target DNA  
5 prepared from 10 different reference strains of *E. coli*. The inventors have shown that the two specific probes on the chip specifically hybridized to the strains of *E. coli* serotype O157:H7.

While the present invention has been described with reference to what are presently considered to be the preferred examples, it  
10 is to be understood that the invention is not limited to the disclosed examples. To the contrary, the invention is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the appended claims.

All publications, patents and patent applications are herein  
15 incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

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**Table 1****DNA sequences of the selective primers that produced the AFLP marker**

Primer	Oligo-nucleotide sequence	
EcoR I-C	5'-GACTGCGTACCAATTCC-3'	(SEQ.ID.NO.:7)
Mse I-G	5'-GATGAGTCCTGAGTAAG-3'	(SEQ.ID.NO.:8)

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**Table 2**  
**E. coli strains screened for the presence**  
**of the O157:H7 marker in AFLP analysis**

Serotype	No. of strains	Serotype	No. of strains	Serotype	No. of strains
O?:H7	1	O91:H21	5	O139:K82	1
O?:H8	1	O91:NM	2	O145:NM	2
O5:NM	4	O98:NM	1	O153:H25	3
O7:H4	4	O103:H2	24	O153:H31	1
O8:H19	1	O111:H8	1	O156:H7	1
O22:H8	5	O111:NM	5	O156:NM	1
O26:H11	6	O113:H4	1	O157:H7	56
O39:H49	1	O113:H21	2	O157:H19	1
O46:H38	1	O115:H8	1	O157:H25	1
O55:H7	3	O118:H16	1	O157:NM	2
O76:H25	1	O121:H7	1	O163:H19	1
O80:NM	3	O127:H6	1	O163:NM	1
O88:H25	1	O128:B12	1		
O91:H14	3	O132:NM	11		

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**Table 3**  
**DNA sequences of the primers specific for**  
**E. coli O157:H7, O157:NM and O55:H7**

Primer	Oligo-nucleotide sequence	Location within the marker sequence
O157-F (22-mer)	5'-CGGTTTAATGGCTTGTTGTGCT-3'	597 - 618 (SEQ.ID.NO.:3)
O157-R (19-mer)	5'-ATGCCATTAAACCGGTGGC-3'	1136 - 1155 (SEQ.ID.NO.:4)

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**Table 4**  
**E. coli strains tested for the presence of the**  
**O157:H7 marker in the PCR assay**

Serotype	No. of strains	Serotype	No. of strains	Serotype	No. of strains
O?:H2	1	O55:H7	6	O126:H8	1
O?:H7	1	O76:H25	1	O127:H6	1
O?:H8	1	O80:NМ	2	O128:B12	1
O?:H19	1	O84:H2	1	O132:NМ	2
O?:H21	1	O88:H25	1	O136:H16	1
O2:H29	1	O91:H14	3	O139:K82	1
O5:NМ	2	O91:H21	3	O142:H38	1
O7:H4	2	O91:NМ	2	O145:NМ	5
O8:H?	1	O98:NМ	1	O153:H25	3
O8:H9	1	O103:H2	5	O153:H31	1
O8:H19	3	O111:H8	2	O153:NМ	1
O15:H27	1	O111:NМ	5	O156:H7	1
O15:NМ	1	O113:H4	1	O156:NМ	1
O22:H8	2	O113:H21	2	O157:H7	15
O26:H11	5	O115:H8	1	O157:H19	1
O26:NМ	1	O116:H21	1	O157:H25	2
O38:H21	1	O118:H16	1	O157:NМ	6
O39:H49	1	O121:H6	1	O163:H19	1
O45:H2	1	O121:H7	2	O163:NМ	2
O46:H38	1	O121:H19	1	O165:NМ	1

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**Table 5**  
**Non-E. coli species tested for the presence**  
**of the O157:H7 marker in the PCR assay**

Species	No. of isolates
<i>Acinetobacter calcoaceticus</i>	1
<i>Aeromonus hydrophila</i>	2
<i>Bacillus cereus</i>	2
<i>Bacillus circuluris</i>	1
<i>Bacillus parabrevis</i>	1
<i>Bacillus subtilis</i>	1
<i>Citrobacter diversus</i>	1
<i>Citrobacter freundii</i>	1
<i>Campylobacter</i>	2
<i>Campylobacter coli</i>	2
<i>Campylobacter jejuni</i>	1
<i>Canadida albicus</i>	1
<i>Enterobacter cloacae</i>	1
<i>Enterobacter amnigenus</i>	1
<i>Enterococcus faecalis</i>	2
<i>Edwardsiella tarda</i>	1
<i>Kluyvera ascovbata</i>	1
<i>Listeria monocytogenes</i>	1
<i>Listeria ivarovii</i>	1
<i>Lactobacillus brevis</i>	1
<i>Lactobacillus planterum</i>	1
<i>Moraxella osloensis</i>	1
<i>Proteus vulgaris</i>	1
<i>Proteus mirabilis</i>	1
<i>Pseudomonas aeruginosa</i>	1
<i>Salmonella typhimurium</i>	4
<i>Salmonella heidelberg</i>	1
<i>Salmonella thompson</i>	1
<i>Salmonella newport</i>	1
<i>Salmonella hadar</i>	2
<i>Salmonella infantis</i>	2
<i>Salmonella schwarzengrund</i>	2
<i>Salmonella choleraesuis</i> var.kunzendorf	2
<i>Shigella flexaeri</i>	1
<i>Shigella sonnei</i>	1
<i>Straphylococcus aureus</i>	4
<i>Straphylococcus epidermidis</i>	1
<i>Straphylococcus haemolyticus</i>	1
<i>Straphylococcus lugdenensis</i>	1
<i>Straphylococcus intermedius</i>	1
<i>Streptococcus pyogenes</i>	1
<i>Streptococcus bovis</i>	1
<i>Xanthomonas malitophila</i>	1
<i>Yersinia enterocolitica</i>	1

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**Table 6**  
**DNA sequences of the probes specific for**  
**E. coli O157:H7, O157:NM and O55:H7**

Probe	Nucleotide sequence	Location within the marker sequence
O157-A	CGGTTTAATGGCTTGTGTG GTAACACCGAAGCCAGCTCA ATAAATTGCTGCGATGAGTT ACAGCTATCGAGTAAACCACC	597 - 677
O157-B	TATCCCGAATCGCCTGGCGT TTTTGCACATC CTCTGCGAC GCTATTTTGTGGAACGCAA AGCCATCAAGGAAAATAGCC ACCGGTTTAATGGCAT	1059 - 1155

**We Claim:**

1. An isolated nucleic acid molecule comprising the sequence shown in Figure 1 (SEQ.ID.NO.:1) or a diagnostic fragment thereof.
2. An isolated nucleic acid molecule comprising (1) a nucleic  
5 sequence shown in Figure 1 (SEQ.ID.NO.:1) wherein T can also be U or a  
diagnostic fragment thereof; (2) a nucleic sequence that is complimentary  
to the sequence shown in Figure 1 (SEQ.ID.NO.:1) or a diagnostic fragment  
thereof; (3) a nucleic sequence that can hybridize to the sequence shown in  
Figure 1 (SEQ.ID.NO.:1) or a diagnostic fragment thereof; (4) a nucleic acid  
10 sequence which has substantial sequence homology to (1), (2) or (3); or (5) a  
nucleic acid sequence which is an analog of any of the nucleic acid  
sequences of (1) to (4).
3. An isolated nucleic acid primer comprising (a) a portion of a  
sequence as claimed in claim 1 or 2 or (b) a sequence that is complimentary  
15 to a portion of a nucleic acid sequence claimed in claim 1 or 2.
4. A nucleic acid primer according to claim 3 wherein the  
primer has the sequence (a) 5'-CGGTTTAATGGCTTGTTGTGCT-3'  
(SEQ.ID.NO.:3) or (b) 5'-ATGCCATTAAACCGGTGGC-3' (SEQ.ID.NO.:4).
5. An isolated nucleotide probe comprising a portion of a  
20 sequence as claimed in claim 1 or 2.
6. A nucleotide probe according to claim 5 comprising  
nucleotides 597-677 as shown in Figure 1 (SEQ.ID.NO.:1).
7. A nucleotide probe according to claim 6 having the sequence  
C G G T T T A A T G G C T T G T T G T G G T A A



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CACCGAAGCCAGCTCAATAAATTGCTGCGATGAGTTAC  
AGCTATCGAGTAAACCACC (SEQ.ID.NO.:5).

8. A nucleotide probe according to claim 7 comprising nucleotides 1059-1155 as shown in Figure 1 (SEQ.ID.NO.:1).

5 9. A nucleotide probe according to claim 8 having the sequence  
T A T C C C G A A T C G C C T G G C G T T T T T G C A C A T C  
CTCTGCGACGCTATTTTTGTGGAACGCAAAGCCATCAAGGAAAATA  
GCCA CCGGTTTAAT GGCAT (SEQ.ID.NO.:6).

10 10. A method of detecting the presence of E. coli serotypes  
O157:H7; O157:NM or O55:H7 in a sample comprising (a) isolating nucleic  
acid from the sample and (b) determining if the isolated nucleic acid  
contains a nucleic acid sequence according to claim 1 or 2, wherein the  
presence of a nucleic acid sequence according to claim 1 or 2 indicates the  
presence of E. coli serotypes O157:H7; O157:NM or O55:H7 in the sample.

15 11. A method of detecting the presence of E. coli serotypes  
O157:H7; O157:NM or O55:H7 in a sample comprising (a) isolating nucleic  
acid from the sample; (b) amplifying the isolated nucleic acid with a  
primer having a sequence that is complimentary to a portion of a nucleic  
acid sequence according to claim 1 or 2 and (c) assaying for amplified  
20 sequences, wherein the presence of an amplified sequence indicates that  
the sample contains E. coli serotypes O157:H7; O157:NM or O55:H7.

12. A method according to claim 11 wherein the nucleic acid is  
amplified in step (b) with a pair of primers, one having a sequence  
5'-CGGTTTAATGGCTTGTGTGCT-3' (SEQ.ID.NO.:3) and the other  
25 having the sequence 5'-ATGCCATTAAACCGGTGGC-3' (SEQ.ID.NO.:4).

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13. A method according to claim 11 or 12 wherein the nucleic acid is amplified in step (b) using a Polymerase Chain Reaction.

14. A method of detecting the presence of a nucleic acid molecule associated with E. coli serotypes O157:H7; O157:NM and O55:H7 in a sample comprising (a) contacting the sample under hybridization conditions with one or more of nucleotide probes which hybridize to nucleic acid molecules according to claim 1 or 2 and (b) determining if there is hybridization between the nucleic acid molecules in the sample and the nucleotide probes, wherein the presence of hybridization indicates that the sample contains E. coli serotypes O157:H7; O157:NM or O55:H7.

15. A method according to claim 14 wherein the nucleotide probe comprises nucleotides 597-677 as shown in Figure 1 (SEQ.ID.NO.:1).

16. A method according to claim 15 wherein the probe has the sequence C G G T T T A A T G G C T T G T T G T G G T A A  
15 C A C C G A A G C C A G C T C A A T A A A T T G C T G C G A T G A G T T A C  
A G C T A T C G A G T A A A C C A C C (SEQ.ID.NO.:5).

17. A method according to claim 15 wherein the nucleotide probe comprises nucleotides 1059-1155 as shown in Figure 1 (SEQ.ID.NO.:1).

18. A method according to claim 17 wherein the probe has the sequence T A T C C C G A A T C G C C T G G C G T T T T T G C A C A T C  
20 C T C T G C G A C G C T A T T T T T G T G G A A C G C A A A G C C A T C A A G G A A A A T A  
G C C A C C G G T T T A A T G G C A T (SEQ.ID.NO.:6).

19. A microchip comprising a nucleic acid molecule according to claim 1 or 2 attached to a microchip.

20. A microchip comprising a nucleotide probe according to any one of claims 5 to 9 attached to a microchip.

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
21 December 2000 (21.12.2000)

PCT

(10) International Publication Number  
**WO 00/77247 A1**

- (51) International Patent Classification<sup>7</sup>: C12Q 1/68 (74) Agent: BERESKIN & PARR; 40th floor, 40 King Street West, Toronto, Ontario M5H 3Y2 (CA).
- (21) International Application Number: PCT/CA00/00716
- (22) International Filing Date: 14 June 2000 (14.06.2000)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data: 60/139,260 15 June 1999 (15.06.1999) US
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- (81) Designated States (national): AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.
- (84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).
- Published:**
- With international search report.
  - Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: A MARKER SPECIFIC FOR *ESCHERICHIA COLI* SEROTYPES O157:H7; O157:NM AND O55:H7

(57) Abstract: A novel DNA marker specific for *E. coli* serotypes O157:H7; O157:NM and O55:H7 is disclosed. The isolation of the marker allows the development of diagnostic assays that can be used to detect the serotypes in the sample. In particular, the marker can be used to prepare nucleic acid primers and nucleotide probes based on the sequence of the marker.

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**FIGURE 1****DNA sequence of the 1583 bp fragment**

CTGCACCTTT TTTTGCTGTG CCTTTGGGAT CCGCGCGAAT GCGCCCAGAA	50
AACCATCAGA AAGTGCCACC TTGGGCGTTT GCACCGTCAT TTGTCACCTC	100
CGGACAGATG TTGCAATGTA TTTTCGTCAA TTGGCCCGAA TGCAACGTGC	150
CAACCCCTGG TTGTGAATAA TGGAATAAAT TCTTGATTAT CAATAATCAA	200
TGCCCATTTT TGTGATGGCC AGGCGAGATC GGCTTCCGCA ATAATTTTAC	250
CTTCATCATT TTGCAATTCG TAGCCGAGAG TCGGCGCAGA GAGAGAATGA	300
GACTGAAGCA ACGCGATCTC TTCAGCGCTT AATAAGCCAA ACTCAATAAT	350
CTCAGCCCAA CTCTCATCAG TCTCTGGTAC TGCCTCTGGC GTTGCCGAAA	400
CTTTAGTGTC AACGCTTTC TCGGTAAAT GCACGGCTTT ACGGCTGGTA	450
AAGGTCATAT CCGGCAGGAA CTGTAACAGG TTTACCAGTC GCCAGAAGCC	500
ATTGAGTCCC GCTTCATAAC CGTCATCCTG CGTATAGCGA TCGTCAAAC	550
AGATATGCAA ACGCAGCCAG CTGCGCATTT GTGCGACGCT GGTCTGCGGT	600
TTAATGGCTT GTTGTGGTAA CACCGAAGCC AGCTCAATAA ATTGCTGCGA	650
TGAGTTACAG CTATCGAGTA AACCACCAA GACAAACGGT TCGTCTGGCA	700
AAAGCTCTGC TAGCCGCCAA GCTGGCGCGT TTTCTGTCAT TTCATAGGCA	750
TACTTTTGTT TCGTTCCCGC GTCCTGTGAC TTCTTCACAT CCAACCACAC	800
CCAGGCATGT GCAGCAGCCA TTCTCTGCCA TAACTGCGTT CTCCGCCCCG	850
GATCGGCGAG ATAATCCAGC AGCAGTGCAA AACTGTTGCG TTCCCGGAAC	900
GACGCTTCAA GGGCCGCAA GTTGGTATCA TGAAACAGGT TATAAACTT	950
AGGCTGTTTC ATATCCGGGT TATGCCCAAG TCCCAGGACA TCCTGAACAT	1000
GTTTAAGCCC TGGCTCTTTG AGATCGGCCA CGTGACTGTC CACACCCAGA	1050
AATTACCGCT ATCCCGAATC GCCTGGCGTT TTTGCACATC CTCTGCGACG	1100
CTATTTTGT GGAACGCAA GCCATCAAGG AAAATAGCCA CCGGTTTAAT	1150
GGCATCAGAC TGCATAAGCG GATATAGCAC GTAATCCGGG CGACAAGGGA	1200
TCCCCACCGC CTCTTTAGCG CCTAAATCCA CTTGAGCTTT AAGATGCCAG	1250
CTCATCATCG GTTCGCTGCG GGTGTTAATA ATCCACCCAG CGCCCTGATG	1300
CGCGTAACTG CGACTGACAA TCAGGTTTTT ATTTTCTTGC AGGCAGCTAA	1350
TAAACCGTTT TTCCAGCTCA CTGCCCATCA TGGCTTCGAG GGAAATGTTT	1400
TTGATGGAAT CAATTACCCG TATCGAATCT CTTGCCTGCA AAATTTTCGC	1450
CAGCAAGAGA CGCGCTGAT CGCGGGAGAC ATACTTCATG CGCCACGGT	1500
CACGGTAAGC GTAAACGCAA CGATAACAAC CATCTTTATG CGTATCATTG	1550
TTGCAACTGC ATTCCACTAT TGCCTGATAG GCC	1583

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**FIGURE 2****DNA sequence of the 360 bp AFLP marker**

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CTGCACCTTT TTTGCTGTG CCTTTGGGAT CCGCGCGAAT GCGCCCAGAA	50
AACCATCAGA AAGTGCCACC TTGGGCGTTT GCACCGTCAT TTGTCACCTC	100
CGGACAGATG TTGCAATGTA TTTTCGTCAA TTGGCCCGAA TGCAACGTGC	150
CAACCCCTGG TTGTGAATAA TGGAATAAAT TCTTGATTAT CAATAATCAA	200
TGCCCATTTT TGTGATGGCC AGGCGAGATC GGCTTCCGCA ATAATTTAC	250
CTTCATCATT TTGCAATTCG TAGCCGAGAG TCGGCGCAGA GAGAGAATGA	300
GACTGAAGCA ACGCGATCTC TTCAGCGCTT AATAAGCCAA ACTCAATAAT	350
CTCAGCCCAA	360

---

**FIGURE 3**

[illegible]

**DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN  
APPLICATION DATA SHEET (37 CFR 1.76)**

As the below named inventor(s), I/we declare that:

This declaration is directed to:

- ☐ The attached application, or  
☒ Application No. PCT/CA00/00716 /, filed on June 14, 2000 /,  
☐ as amended on \_\_\_\_\_ (if applicable);

I/we believe that I/we am/are the original and first inventor(s) of the subject matter which is claimed and for which a patent is sought;

I/ we have reviewed and understand the contents of the above-identified application, including the claims, as amended by any amendment specifically referred to above;

I/we acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me/us to be material to patentability as defined in 37 CFR 1.56, including material information which became available between the filing date of the prior application and the National or PCT International filing date of the continuation-in-part application, if applicable; and

All statements made herein of my/own knowledge are true, all statements made herein on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001, and may jeopardize the validity of the application or any patent issuing thereon.

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☐ Additional inventors are being named on \_\_\_\_\_ additional form(s) attached hereto.

Burden Hour Statement: This collection of information is required by 35 U.S.C. 115 and 37 CFR 1.63. The information is used by the public to file (and the PTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This form is estimated to take 1 minute to complete. This time will vary depending upon the needs of the individual case. Any comments on the amount of time you are required to complete this form should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, Washington, DC 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Assistant Commissioner for Patents, Washington, DC 20231.



## Supplemental Application Data Sheet

### Application Information

Application Type:: Regular

Subject Matter:: Utility

Suggested  
Classification::

Suggested Group Art  
Unit::

CD-ROM or CD-R?:: None

Number of CD disks:: 0

Number of copies of CDs:: 0

Sequence submission?:: YES

Computer Readable  
Form (CRF)?:: YES

Number of copies of CRF:: 1

Title:: A MARKER SEPCIFIC FOR *ESCHERICHIA COLI*  
SEROTYPES O157:H7; O157:NM AND O55:H7

Attorney Docket Number:: 6580-273

Request for Early  
Publication?:: No

Request for Non-Publication?:: No

Suggested Drawing Figure:: 2

Total Drawing Sheets:: 3

Small Entity?:: Yes

Latin Name::

Variety denomination  
name::

Petition included?:: No

Petition Type::

Licensed US Govt.  
Agency::

Contract or Grant  
Numbers::

Secrecy Order in  
Parent Appl.?:: No

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### **Representative Information**

Representative  
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001059

### Domestic Priority Information

Application::	Continuity Type::	Parent Application::	Parent Filing Date::
This application PCT/CA00/00716	371 of Non-Provisional of	<u>PCT/CA00/00716</u> <u>60/139,260</u>	<u>June 14, 2000</u> <u>June 15, 1999</u>

### Foreign Priority Applications

Country::	Application Number::	Filing Date::	Priority Claimed
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1/2

## SEQUENCE LISTING

<110> Chen, Shu  
 Xu, Renlin  
 LI, Jiping  
 University of Guelph

<120> A Marker Specific for Escherichia coli Serotypes  
 O157:H7; O157:NM and O55:H7

<130> 6580-201

<140>

<141>

<160> 8

<170> PatentIn Ver. 2.0

<210> 1

<211> 1583

<212> DNA

<213> E.coli

<400> 1

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ttttcgtcaa ttggcccga a tgcaacgtgc caaccctgg ttgtgaataa tggaataaat 180
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